Brief Definitive Report

Role of Dok-1 and Dok-2 in Leukemia Suppression

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Abstract

Chronic myelogenous leukemia (CML) is characterized by the presence of the chimeric p210bcr/abl oncprotein that shows elevated and constitutive protein tyrosine kinase activity relative to the normal c-abl tyrosine kinase. Although several p210bcr/abl substrates have been identified, their relevance in the pathogenesis of the disease is unclear. We have identified a family of proteins, Dok (downstream of tyrosine kinase), coexpressed in hematopoietic progenitor cells. Members of this family such as p62dok (Dok-1) and p56dok-2 (Dok-2) associate with the p120 rasGTPase-activating protein (rasGAP) upon phosphorylation by p210bcr/abl as well as receptor and nonreceptor tyrosine kinases. Here, we report the generation and characterization of single and double Dok-1 or Dok-2 knockout (KO) mutants. Single KO mice displayed normal steady-state hematopoiesis. By contrast, concomitant Dok-1 and Dok-2 inactivation resulted in aberrant hemopoiesis and Ras/MAP kinase activation. Strikingly, all Dok-1/Dok-2 double KO mutants spontaneously developed transplantable CML-like myeloproliferative disease due to increased cellular proliferation and reduced apoptosis. Furthermore, Dok-1 or Dok-2 inactivation markedly accelerated leukemia and blastic crisis onset in Tec-p210bcr/abl transgenic mice known to develop, after long latency, a myeloproliferative disorder resembling human CML. These findings unravel the critical and unexpected role of Dok-1 and Dok-2 in tumor suppression and control of the hematopoietic compartment homeostasis.

Key words: cell proliferation • apoptosis • knockout • CML leukemogenesis • signal transduction

Introduction

Chronic myelogenous leukemia (CML) is a clonal disorder of the hematopoietic cells characterized by the presence of the Philadelphia chromosome (Ph+), which is the result of a chromosomal translocation between the BCR gene on chromosome 22 and the ABL gene on chromosome 9 (1, 2). A bcr-abl chimeric protein originates from this translocation. Its p210 form, which is the causative mutation found in 95% of cases of CML, has elevated tyrosine kinase activity and exists exclusively in cytoplasm compared with endogenous c-ABL (1, 2). Two phases of the disease have been characterized: (a) a chronic phase with an average span of 3–5 yr during which the Ph+ cells populate the entire intermediate and late hematopoietic maturational compartments, and (b) an acute malignant and fatal stage known as blast crisis when the leukemic cells acquire additional genetic changes, lose their ability to differentiate and mature, and acquire the ability to infiltrate and colonize other organs (1, 2). Inhibition of p210bcr/abl activity by selective drugs such as STI571 leads to disease remission, making CML a paradigmatic example of targeted cancer therapy (3). However, patients do relapse upon STI571 treatment, underscoring the need to identify critical downstream events in the p210bcr/abl signaling cascade. Furthermore, the genetics of...
blastic crisis transformation is poorly understood. p66\textsuperscript{Dok-2}\textsuperscript{\textcircled{2}}
(Dok-1) was cloned as a major phosphorylation substrate of the p21\textsuperscript{Fcr/\textcircled{1}} oncoprotein in Ph\textsuperscript{+} CML blasts, as well as a major substrate of many tyrosine kinases (4, 5). Soon after, additional members of the family (6–9) have been identified. These proteins resemble docking proteins in their structure because they contain PH and PTB domains as well as multiple binding motifs for SH2 and SH3 domains. Three of them (Dok-1–3) are differentially expressed in the hematopoietic compartment, coexpressed in the hematopoietic progenitors, and aberrantly phosphorylated by p21\textsuperscript{Fcr/\textcircled{1}} (4, 6, 7, 9). Several recent reports implicate Dok proteins in the negative regulation of signaling pathways activated by tyrosine kinases (9–14). Unlike Dok-3–5, Dok-1 and Dok-2 are able to associate with rasGAP when phosphorylated, suggesting that they may serve critical, but possibly redundant, functions (6, 9). Therefore, to determine the role of DOK as p21\textsuperscript{Fcr/\textcircled{1}} substrates in hematopoiesis and CML pathogenesis, we studied in vivo in the mouse the effect of combined inactivation of DOK family members. Here, we unravel the key tumor suppressive role of Dok-1 and Dok-2 in the hematopoietic compartment and their importance in CML pathogenesis.

Materials and Methods

**Targeting Vector and Generation of Dok-2\textsuperscript{\textcircled{+}/\textcircled{–}} Embryonic Stem Cells.** A 129/Sv mouse genomic library (Stratagene) was screened with a probe containing murine Dok-2 exon 1. Exon/intron boundaries of the isolated Dok-2 genomic clones were determined by restriction enzyme mapping, DNA sequencing, and PCR. To generate the targeting construct, a 2.7-Kb EcoRI–SacI genomic fragment (5′ arm) and a 4.5-Kb Xhol–HindIII genomic fragment (3′ arm) were cloned into the pPNT vector (13). The targeting construct was linearized with NotI and electroporated into C57 embryonic stem cells. Transfectedants were selected in 350 μg/ml G418 and 2 μM gancyclovir and expanded for Southern blot analysis using a 5′ probe (see Fig. S1 A below).

**Generation of Dok-2\textsuperscript{\textcircled{+}/\textcircled{–}}, Double KO (DKO), and Tcc-p210\textsuperscript{Fcr/\textcircled{1}}/Dok-1/Dok-2 Compound Mutants.** Chimeric mice and F1 offspring were produced as described previously (13). Chimeric males were then mated with 129/Sv females (The Jackson Laboratory) to obtain Dok-2 mutants in a 129/Sv background. Dok-1\textsuperscript{–/–} mice (13) and Tcc-p210\textsuperscript{Fcr/\textcircled{1}}/Dok-1/Dok-2 compound mice were obtained by interbreeding Dok-1\textsuperscript{–/–} mice with Dok-2\textsuperscript{–/–} mice both in 129/Sv background. To obtain Tcc-p210\textsuperscript{Fcr/\textcircled{1}}/Dok-1/Dok-2 compound mice, Tcc-p210\textsuperscript{Fcr/\textcircled{1}}/Dok-1/Dok-2 compound mice were first crossed with Dok-1\textsuperscript{–/–} mice (129/Sv) or Dok-2\textsuperscript{–/–} mice (129/Sv). F1 offspring were then mated with each other to get Tcc-p210\textsuperscript{Fcr/\textcircled{1}}/Dok-1/Dok-2 null mice and to balance the genetic background. All mice studies were approved by the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center.

**Follow-up Design and Leukemia Diagnosis.** Mice were monitored monthly by peripheral blood (PB) counts and smears (bi-weekly in the case of Tcc-p210\textsuperscript{Fcr/\textcircled{1}}/Dok-1/Dok-2 compound mutants and BM transplantation). Diagnosis of leukemia was made on the criteria that two consecutive white blood cell counts are \(>20\times10^3/\muL\). Autopsies were performed on dead or moribund animals as described previously (17). For B220 or CD3 detection, immunohistochemistry was performed on representative sections using anti-mouse B220 monoclonal antibody (RA3-6B2; BD Biosciences) or a rabbit anti-CD3 polyclonal antibody (DakoCytomation) according to the manufacturer’s instructions.

**BM Transplantation.** 2 \(\times\) 10\(^6\) BM cells from WT or Dok-1\textsuperscript{–/–}/Dok-2\textsuperscript{–/–} mutant mice were injected via tail vein into lethally irradiated (920 rads) 129/Sv WT mice (6-wk-old female). Recipient mice were monitored and scored positive for disease according to criteria mentioned in the legend to Fig. S2 (see below).

**Western Blot and Flow Cytometric Analysis.** These analyses were performed as described previously (13, 18). For Western blot analysis, we used a rabbit polyclonal anti–Dok-R/Dok2 antibody (Upstate Cell Signaling) to detect Dok-2 protein. To detect Erk 2 protein, we used a polyclonal anti–Erk 2 antibody (Santa Cruz Biotechnology, Inc.). For flow cytometry, we used the following conjugated antibodies: anti–c–Kit, anti–Sca-1, anti–Mac-1, anti–Gr-1, anti–F4/80, anti–CD3 complex, anti–B220, anti–CD4, and anti–CD38. Anti–F4/80 was obtained from CalTag. All other antibodies were from BD Biosciences. Flow cytometry was performed using a FACScan (Becton Dickinson).

The data were analyzed using FlowJo software (Tree Star).

**Ras GTPase Activation Assay.** Activation of Ras was measured using GST-RBD (Ras-binding domain of Raf/RBD) pull down assays (19). The underlying premise of this assay is that the RBD binds only to GTP-bound Ras proteins. Mac-1\textsuperscript{–/–} cells were isolated from freshly isolated BM cells using CD11b Microbeads (Miltenyi Biotec). Purity (>90%) was confirmed by flow cytometry. Mac-1\textsuperscript{–/–} cells were incubated in RPMI/0.1% FCS for 3 h, and then stimulated with 10 ng/ml GM-CSF for 10 min at 37°C. Cells were then lysed in 25 mM Hepes, pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 25 mM NaF, 10 mM MgCl\(_2\), 1 mM EDTA, 2.5 mM sodium deoxycholic, and 1 mM Na\(_2\)VO\(_4\) plus protease inhibitors. An equal amount of cell lysates was incubated with GST-RBD coupled to glutathione beads. Bead-associated Ras (GTP-bound Ras) and total Ras in cell lysates were detected by Western blotting with an anti–pan-Ras antibody (Transduction Laboratories). GM-CSF–induced Ras activation is measured by normalizing the amount of GTP-bound Ras to the total amount of Ras in cell lysates.

**Proliferation, Apoptosis, and In Vitro Colony-forming Assay.** BM cells were flushed from murine femurs and tibiae. 2 \(\times\) 10\(^6\) cells were plated in MethoCult M3434 (StemCell Technologies Inc.). Colonies were counted on days 2 (CFU-E), 7 (CFU-GM and BFU-E), and 13 (CFU-GEMM). For proliferation assay of collected cells from in vitro colony-forming assay, 2 \(\times\) 10\(^7\) BM cells were plated in MethoCult M3234 with 20 ng/ml IL-3, 50 ng/ml G-CSF, and 20 ng/ml GM-CSF. At day 7, cells were collected from methylcellulose and washed by RPMI/10% FCS and counted. 2.5 \(\times\) 10\(^6\) cells were then added for 4 h. For proliferation assays of BM cells, contaminating erythrocytes were removed by hypotonic lysis. Mac-1\textsuperscript{+} cells were isolated using CD11b Microbeads (Miltenyi Biotec). Purity (>90%) was confirmed by flow cytometry. Cells were treated with 10 ng/ml IL-3, 10 ng/ml stem cell factor (SCF), or 10 ng/ml GM-CSF for 24 h. [\(^3\)H]thymidine was then added for 6 h. For apoptosis analysis, BM cells were cultured as described above. After 48 h, cells were harvested and incubated with anti–CD16/32 to block nonspecific binding. Cells were then stained with anti–Mac-1–APC and annexin V (BD Biosciences) according to the manufacturer’s instructions. The percentage of apoptotic cells was determined by FACS analysis (FACSCalibur; Becton Dickinson).

**Spectral Karyotyping Analysis.** BM cells from DKO mice were cultured in RPMI 1640/10% FCS with 6 ng/ml IL-3, 10 ng/ml IL-6, 100 ng/ml SCF, and 10 ng/ml BrdU for 18 h. The cells
were then prepared for cytogenetic analysis performed using a mouse SkyPaint Kit (Applied Spectral Imaging) according to the manufacturer's instructions.

Online Supplemental Material. Fig. S1 shows targeted disruption of the Dok-2 gene. Fig. S2 shows results of adoptive transfer of BM cells from DKO mice with myeloproliferative disease (MPD). Fig. S3 shows FACS analysis of BM and spleen cells from leukemic Tec-p210\textsubscript{\textit{bcr/abl}}/Tec-p210\textsubscript{\textit{bcr/abl}}/Dok-1\textsuperscript{--/--}, and Tec-p210\textsubscript{\textit{bcr/abl}}/Dok-2\textsuperscript{--/--} mice and survival curves of these compound mutants. Table S1 shows the data of PB cell count. Figs. S1–S3 and Table S1 are available at http://www.jem.org/cgi/content/full/jem.20041306/DC1.

Results and Discussion

We inactivated the Dok-2 gene in the mouse and crossed Dok-2 mutants with Dok-1\textsuperscript{--/--} mice that we described previously (13). As in the case of Dok-1-targeted disruption, Dok-2\textsuperscript{--/--} mutants were born following mendelian frequen-
cies. Postmortem pathological analysis and monthly flow cytometric and morphological assessments of the various hematopoietic organs from Dok-2−/− mutants revealed normal steady-state hematopoiesis and organogenesis (unpublished data). Analysis of Dok-1 and Dok-2 protein expression in the various hematopoietic organs from Dok-1−/− and Dok-2−/− mutants, respectively, did not reveal compensatory up-regulation of the remaining Dok protein (Fig. S1 E, available at http://www.jem.org/cgi/content/full/jem.20041306/DC1). Furthermore, in standard colony-forming assays in methylcellulose, BM progenitors from Dok-2−/− mutants yielded numbers of erythroid and myeloid colonies comparable to WT sex- and age-matched littermates (Fig. S1 F). Although myeloid differentiation was up-regulated in the various hematopoietic organs from single Dok-1 and Dok-2 mutants, respectively, did not reveal compensatory up-regulation of the remaining Dok protein (Fig. S1 E, available at http://www.jem.org/cgi/content/full/jem.20041306/DC1). Furthermore, in standard colony-forming assays in methylcellulose, BM progenitors from Dok-2−/− mutants yielded numbers of erythroid and myeloid colonies comparable to WT sex- and age-matched littermates (Fig. S1 F). Although myeloid differentiation was overall normal in Dok-2−/− mice, we did observe a consistent defect in the activation of Dok-2−/− mature granulocyte upon TPA treatment (unpublished data). Nevertheless, Dok-2−/− mice did not display an increase incidence of spontaneous infections. The fact that both Dok-1 and Dok-2 KO mutants displayed normal steady-state hemopoiesis suggested that they may exert redundant function.

Therefore, we generated DKO mutants. These mutants were also developmentally normal (as revealed by pathological analysis of all organs; unpublished data) and fertile. However, monthly postmortem pathological, flow cytometric, and morphological assessments of the various hematopoietic organs from DKO mutants unraveled striking differences with respect to the Dok-1 and Dok-2 single KO mutants. In fact, DKO mutants developed at complete penetrance a CML-like MPD at 10–12 mo of age (Fig. 1 A; refer to Materials and Methods). DKO displayed a progressive increase in white blood cell counts in the PB after 4 mo of age (Fig. 1 B and Table S1, which is available at http://www.jem.org/cgi/content/full/jem.20041306/DC1; at 4 mo: WT [n = 6] 9,800 ± 2,078, DKO [n = 6] 9,725 ± 3,007; at 8 mo: WT [n = 6] 10,033 ± 1,286, DKO [n = 7] 15,575 ± 2,326; at 12 mo: WT [n = 6] 9,200 ± 3,268, DKO [n = 8] 24,180 ± 4,540). At leukemia onset, DKO mice invariably displayed a marked splenomegaly (Fig. 1 C; WT [n = 3] 0.051 ± 0.005 g, DKO [n = 3] 0.081 ± 0.02 g) as well as PB and BM hypercellularity (Fig. 1 D and Table S1). BM and spleen were both predominantly infiltrated by myeloid cells that retained the ability to terminally differentiate (Fig. 1 D). Automated and differential counts in the PB revealed a marked leukocytosis caused by an increase in the number of neutrophils and monocytes (Table S1). Interestingly, erythrocytes and platelets number counts remained relatively normal at this stage (Table S1). The increase in PB cellularity was accompanied by the appearance of undifferentiated blasts in the

Figure 2. Analysis of preleukemic Dok-1−/−/Dok-2−/− mutants. (A) In vitro colony-forming assay performed with BM cells isolated from a WT (white bar) and a Dok-1−/−/Dok-2−/− mutant (DKO; black bar) at 4 mo of age. P-value is also shown. (B) [3H]thymidine incorporation analysis of proliferative response of cells collected from in vitro colony assay of BM cells from 2-mo-old WT, Dok-1−/−, Dok-2−/−, and DKO mice. (C) Proliferative response determined by [3H]thymidine incorporation of Mac-1+ BM cells upon IL-3, GM-CSF, and SCF stimulation. (D) Apoptotic response to growth factor deprivation in BM cells. Cell death was analyzed by annexin V staining of WT (white bar) and Dok-1−/−/Dok-2−/− (DKO; black bar) Mac-1+ BM cells. A representative Western blot from three experiments is shown. Very low amounts of Ras-GTP were detected in serum-starved cells before GM-CSF treatment. (F) Relative Ras activity in the above cells treated with GM-CSF is quantified by the ratio of GTP-bound Ras over total Ras. The value of WT BM cells was set as 1 and the ratios of other types of BM cells were calculated correspondingly. (G) Levels of ERK 1/2 phosphorylation upon (lanes 3 and 4) or in the absence (lanes 1 and 2) of GM-CSF stimulation in BM cells from WT (lanes 1 and 3) and Dok-1−/−/Dok-2−/− (lanes 2 and 4) mice.
Flow cytometric analysis of PB, BM, and spleen confirmed the expansion of the differentiat-
ated myeloid compartment (increase in the percentage of Mac-1⁺, Gr-1⁺, and Mac-1⁺ F4/80⁺ cells with a con-
comitant decrease in the percentage of B220⁺ [B cell] and CD3⁺ [T cell] cells) as well as the presence of undifferenti-
cated cells in the PB (Sca-1⁺ cells: WT [n = 4] 6.5 ± 4.3%, DKO [n = 4] 13.5 ± 2.9%; Fig. 1, F–H). Colony-forming
assays in methylcellulose from BM cells of DKO diseased mice revealed the expansion of myeloid progenitors (Fig. 1
I). A higher number of progenitors (CFU-GM) from BM cells of DKO mice revealed the expansion of myeloid progenitors (Fig. 1
J). As Dok-1 and Dok-2 can act as negative Ras regulator
sequences of concomitant
Figure 3. Leukemia onset and phenotype of Tec-
p210bcr/abl/Dok-1/Dok-2 compound mutants. (A) Leuke-
free survival curves of Tec-p210bcr/abl(+/+), Dok-1
mutants. P-value is also indicated. (B) Leukemia-free
survival curves of Tec-p210bcr/abl(+/+)
Dok-2 mutants. P-value is also indicated. (C) Morphology of PB cells
from a Tec-p210bcr/abl(+/+) DKO mouse during the
blast crisis. Blasts (arrowheads) are indicated. Original
magnification of 200. (D) Magnification (1,000) of
boxed region in C. Blast cell is indicated by arrowhead.
(E) Hematoxylin and eosin staining of lung from a Tec-
p210bcr/abl(+/+) DKO mouse killed during the
blast crisis. Alveolar interstitial spaces are infiltrated by
accumulation of erythrocytes (arrowheads) and invasion of
both myeloid blasts and differentiated cells. Original
magnification of 400. (G) Immunohistochemical staining of
invasive lymphoma in the ileum in a Tec-
p210bcr/abl(+/+) DKO mouse. A monomorphic in-
filtrate of lymphocytic cells (indicated by the dashed oval)
with hyperchromatic and dysplastic nuclei is identified
under the mucosa (arrowheads). These cells are invad-
ing beyond the muscularis mucosa into the submucosa
(stars). Cobble cells (arrow) are seen. Original
magnification of 400. (H and I) Immunohistochemical staining of
the lymphoma shown in G using anti-B220 antibody
(H) for B cell identification) and anti-CD3 antibody (I, for
T cell identification). Original magnification of 200. (J)
Incidence of invasive lymphoma in Tec-p210bcr/abl(+/+) Tec-
p210bcr/abl(+/+) DKO 1⁻/-, and Tec-p210bcr/abl(+/+) DKO 2⁻/-
mice.
p210
\textsuperscript{bcr/abl} TM with Dok-1\textsuperscript{−/−} and Dok-2\textsuperscript{−/−} mutants and assessed whether their inactivation would impact on the biology of the disease. Inactivation of either Dok-1 or Dok-2 accelerated chronic phase onset in compound mutants (Fig. 3, A and B). By contrast, the distinctive features of the chronic phase in Tec-p210
\textsuperscript{bcr/abl} TM were not perturbed by Dok-1 or Dok-2 inactivation as revealed by comparable flow cytometric and morphological profiles of the major hematopoietic organs (Fig. S3 A and unpublished data). Furthermore, and importantly, Dok-1 or Dok-2 inactivation accelerated the onset of the fatal blastic phase of the disease resulting in a marked reduction in overall survival in the compound mutants (Fig. 3, C–F, and Fig. S2, B and C; mean survival of Dok-1 crosses: Tec-p210
\textsuperscript{bcr/abl}/WT: 324.7 ± 50.0 d; Tec-p210
\textsuperscript{bcr/abl}/Dok-1\textsuperscript{−/−}: 307.4 ± 67.1 d; Tec-p210
\textsuperscript{bcr/abl}/Dok-1\textsuperscript{−/−}: 284.9 ± 65.4 d; mean survival of Dok-2 crosses: Tec-p210
\textsuperscript{bcr/abl}/WT: 320.9 ± 54.8 d; Tec-p210
\textsuperscript{bcr/abl}/Dok-2\textsuperscript{−/−}: 282.7 ± 96.0 d; p210
\textsuperscript{bcr/abl}/Dok-2\textsuperscript{−/−}: 270.5 ± 48.7 d) as well as in shortening of the chronic phase (Dok-1 crosses: Tec-p210
\textsuperscript{bcr/abl}/WT [n = 10] 84.6 ± 46.3 d; Tec-p210
\textsuperscript{bcr/abl}/Dok-1\textsuperscript{−/−} [n = 21] 78.2 ± 47.5 d; Tec-p210
\textsuperscript{bcr/abl}/Dok-1\textsuperscript{−/−} [n = 9] 78.2 ± 44.1 d; Dok-2 crosses: Tec-p210
\textsuperscript{bcr/abl}/WT [n = 8] 83.8 ± 31.1 d; Tec-p210
\textsuperscript{bcr/abl}/Dok-2\textsuperscript{−/−} [n = 13] 76.9 ± 39.6 d; Tec-p210
\textsuperscript{bcr/abl}/Dok-2\textsuperscript{−/−} [n = 9] 70.0 ± 27.7 d). Postmortem analysis of compound mutants in various genotypes did not reveal qualitative differences in the biology and cellularity of the hematopoietic organs (Fig. S3 A and unpublished data).

In memory of Hisamaru Hirai.

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References

\textsuperscript{+} leukemia in mice. Oncogene. 21:8643–8651.
\textsuperscript{dok} defines a new family of RasGAP-binding proteins. J. Biol. Chem. 273:4827–4830.
\textsuperscript{dok} as a cytokine-inducible inhibitor of cell proliferation and signal transduction. EMBO J. 19:5114–5122.
\textsuperscript{dok} in negative regulation of B cell receptor-mediated signaling. Genes Dev. 14:11–16.
\textsuperscript{dok}, a negative regulator of Ras and mitogen-activated protein kinase (MAPK) activity, opposes leukemogenesis by p210
\textsuperscript{bcr/abl}. J. Exp. Med. 194:275–284.
\textsuperscript{dok} is essential for its negative effect on mitogen-activated protein (MAP) kinase activation. J. Exp. Med. 194:265–274.


